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Monoclonal Antibodies Produced by Muscle after Plasmid Injection and Electroporation

Torunn Elisabeth Tjelle,[1,*] Alexandre Corthay,[2] Elin Lunde,[3] Inger Sandlie,[3] Terje E. Michaelsen,[4,5] Iacob Mathiesen,[1] and Bjarne Bogen[2,*]

[1] *Inovio AS, Forskningsveien 2a, 0373 Oslo, Norway*
[2] *Institute of Immunology, Rikshospitalet, University of Oslo, Oslo, Norway*
[3] *Institute of Biology, University of Oslo, Oslo, Norway*
[4] *Norwegian Institute of Public Health, Oslo, Norway*
[5] *Institute of Pharmacy, University of Oslo, Oslo, Norway*

To whom correspondence and reprint requests should be addressed. E-mail: t.e.tjelle@inovio.com or bjarne.bogen@labmed.uio.no.

Antibodies are useful for the treatment of a variety of diseases. We here demonstrate that mouse muscle produced monoclonal antibodies (mAb) after a single injection of immunoglobulin genes as plasmid DNA. *In vivo* electroporation of muscle greatly enhanced antibody production. For chimeric antibodies, levels of 50–200 ng mAb/ml serum were obtained but levels declined after 7–14 days due to an immune response against the xenogeneic parts of the antibody. By contrast, fully mouse antibodies persisted in serum for at least 7 months. mAb produced by the muscle had correct structure, specificity, and biological effector functions. The findings were extended to a larger animal, the sheep, in which mAb serum levels of 30–50 ng/ml were obtained. Sustained levels of serum mAb, induced by single injection of Ig genes and electroporation of muscle cells, may offer significant advantages in the treatment of human diseases.

Key Words: antibody therapy, electroporation, nonviral DNA delivery

INTRODUCTION

Injection of DNA that encodes foreign protein induces an immune response and represents an attractive strategy for active vaccination (for review see [1]). However, if the injected DNA encodes a self protein, the immune system should not respond and the self protein might persist in the body for long periods of time [2]. We have here addressed if injection of self immunoglobulin genes can be used for prolonged expression of therapeutic monoclonal antibodies (mAb) in an individual.

Monoclonal antibodies are increasingly being used in treatment of cancer, e.g., anti-CD20 mAb for B lymphomas [3] and anti-Her2 mAb for breast cancer [4]. mAbs are also injected to neutralize tumor necrosis factor α (TNFα) in autoimmune diseases like rheumatoid arthritis and Crohn disease [5]. Moreover, mAbs against CD3 and other T cell markers are useful to reverse rejection of transplants [6]. Finally, mAbs may be used to protect against infectious diseases in immunocompromised individuals, like RSV-infected premature infants [7]. mAbs exert their functions by a variety of mechanisms such as induction of apoptosis, activation of complement, opsonization of target cells for phagocytosis, and neutralization of viruses or inflammatory cytokines.

Although mAbs are increasingly being used in the clinic, such treatments have several shortcomings. As proteins with a limited life span, mAbs need to be injected repeatedly. Moreover, large-scale production of mAbs *in vitro* is technically difficult and expensive and carries a risk of infection. In addition, use of animal cell lines for production may introduce non-human posttranslational modifications in the mAbs [8].

Efforts have been made to overcome these problems by injecting animals with cells that have previously been transfected in vitro with immunoglobulin (Ig) genes. Thus, transfected myogenic cells [9], skin fibroblasts [10], or keratinocytes [11] have been used to obtain prolonged expression of mAbs. This strategy has the disadvantage that cells have to be grown *ex vivo*, transfected, and introduced into the individual, which is a lengthy, costly procedure with a risk of contamination. Recently, recombinant adeno-associated virus [12] and adenovirus [13] vectors were used to deliver the genes encoding a human anti-HIV mAb [12] or an anti-human thyroglobulin mAb [13] to mouse muscle cells *in vivo*. Serum levels in the μg/ml range were obtained and mAbs persisted in the serum for months. Although these results are very encouraging, the use of virus as a vehicle may

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cause adverse side effects such as induction of an antiviral immune response [13,14], integration of viral DNA in chromosomal DNA [15] even in germ cells [16], and possible induction of cancer [17]. In addition, it is still difficult and expensive to produce large amounts of virus vector for clinical use. Plasmids are considered to be safer than viral vectors; however, it is difficult to rule out that plasmids might randomly insert into the genome with possible deleterious effects such as cancer development.

Bearing the aforementioned concerns in mind, we sought to develop a simple way of obtaining sustained high-level production of mAb by muscle cells after injection of Ig genes as naked plasmids *in vivo*. We and others have previously shown that electroporation enhances the gene transfection efficiency of mouse and rat muscle more than 100-fold without detrimental side effects [18–20]. Electroporation is the application of low-voltage pulses to muscle to destabilize the cell membrane and thereby allow macromolecules such as plasmids to enter the cells more efficiently. Here, we have injected naked plasmid DNA encoding heavy (H) and light (L) chains of mAb into skeletal muscles of mice and sheep, followed immediately by *in vivo* electroporation of muscle cells. The results demonstrate that sustained expression of high serum levels of functional mAbs is obtained by this approach in both small and larger animals.

RESULTS

In Vivo Electroporation Enhances Monoclonal Antibody Production after Intramuscular Injection of Ig Genes Residing on a Single or on Two Separate Plasmids

We first tested if electroporation could enhance expression of H- and L-chain Ig genes injected intramuscularly as naked DNA plasmids into the quadriceps muscles of C57Bl/6 mice. The Ig genes were under the control of a cytomegalovirus (CMV) promoter and encoded a chimeric mouse–human IgG3 with specificity for a mouse major histocompatibility complex (MHC) class II molecule, I-E^d. Co-injection of separate plasmids for H- and L-chain genes, as well as injection of a single "combi" plasmid containing both genes (Table 1), induced only low amounts of serum mAb not significantly higher than in control mice treated with electroporation but no DNA (Fig. 1A). However, levels of serum mAb increased considerably (Fig. 1A) when injection of plasmid DNA was followed by *in vivo* electroporation consisting of low-voltage, high-frequency electrical pulses applied by caliper electrodes to the muscle through the skin above the injection site. Thus, electroporation greatly enhanced production of mAbs from Ig genes injected as naked DNA plasmids. Moreover, as injection of two plasmids gave results similar to those of the combi plasmid, Ig H- and L- chain genes did not need to be joined.

Table 1: Recombinant mAb and DNA plasmids that encode them

Specificity mAb[a]	Donor V regions[b] (B cell hybridoma)	C regions[c]	Name of plasmid	Ref. plasmids
I-E^d	14-4-4S (Ref. [33])	hCγ3	pLNOH2 $\gamma 3V_HS^{d,e}$	[31]
		hCκ	pLNOκ V_LS	[31]
		hCγ3	pLNOH2 $\gamma 3V_HT$	[30]
IgD[a]	Ig(5a)7.2 (Ref. [34])	hCκ	pLNOκ V_LT	[30]
		mγ2b	pLNOH2 $\gamma 2bV_HT$	Footnote[f]
		hCκ	pLNOκ V_LT	[30]
NIP	B1-8 (Ref. [35])	mγ2b	pLNOH2 $\gamma 2bV_H$NIP	[29]
		mλ	$\lambda 1$	Celltech Limited

[a] I-E is a mouse MHC class II molecule, I-E^d is the allotype encoded by the H-2^d haplotype. IgD is an Ig isotype used as B cell receptor for antigen on B cell. IgD^a is a particular allotype of IgD encoded by the IgH^a haplotype. NIP is the hapten 4-hydroxy-3iodo-5-nitrophenyl-acetic acid.

[b] All V regions are of mouse origin.

[c] "m" denotes mouse, "h" denotes human.

[d] In this particular plasmid, the H chain had a 91-101 $\lambda 2^{315}$ T cell epitope introduced into its C region. This epitope does not influence secretion and folding if the mAb [31].

[e] For I-E^d-specific mAb, a single "combi" vector that contains both the H and the L chain genes [31] was also used.

[f] The pLNOH2γ2bV_HT vector was constructed by replacing the hγ3 constant region gene of pLNOH2-$\gamma 3V_HT$ with mγ2b [29,30] (unpublished).

Monoclonal Antibodies Produced by Injection of Ig Genes and Electroporation Have the Expected Specificity and Are Absorbed by Their Targets *in Vivo*

It was important to demonstrate that mAbs produced by plasmid injection and electroporation had the expected antigen binding specificity for their targets *in vivo*. To this end, we took advantage of the specificity of the mAb for I-E^d; this is a MHC class II allotype that is not expressed by C57Bl/6 mice but is expressed by certain other strains like BALB/c. Accordingly, mAb produced in BALB/c mice, but not in C57Bl/6 mice, should be rapidly absorbed by the I-E^d-positive tissue. Indeed, following DNA injection of the combi H+L plasmid and electroporation, we detected the mAb easily in C57Bl/6 sera but not in BALB/c sera (Fig. 1B). An alternative explanation for this finding could be that BALB/c mice, due to their different genetic make-up compared to C57Bl/6 mice, did not respond to the DNA injection/electroporation procedure. Excluding this possibility, BALB.B mice, which are very similar to BALB/c but lack I-E^d, had easily detectable mAb in their sera (Fig. 1C). Likewise, in B10.D2 mice, which are very similar to C57Bl/6 but express I-E^d, we could detect no mAb in serum (Fig. 1C).

We repeated the experiments with another chimeric IgG3 mAb with human constant regions but with mouse V regions specific for an allotype of mouse IgD, namely IgD^a. In this case we injected two separate plasmids, encoding the H and L chains respectively (Table 1). As can be seen from Fig. 1D, co-injection of plasmids resulted in detection of human IgG3 in sera of

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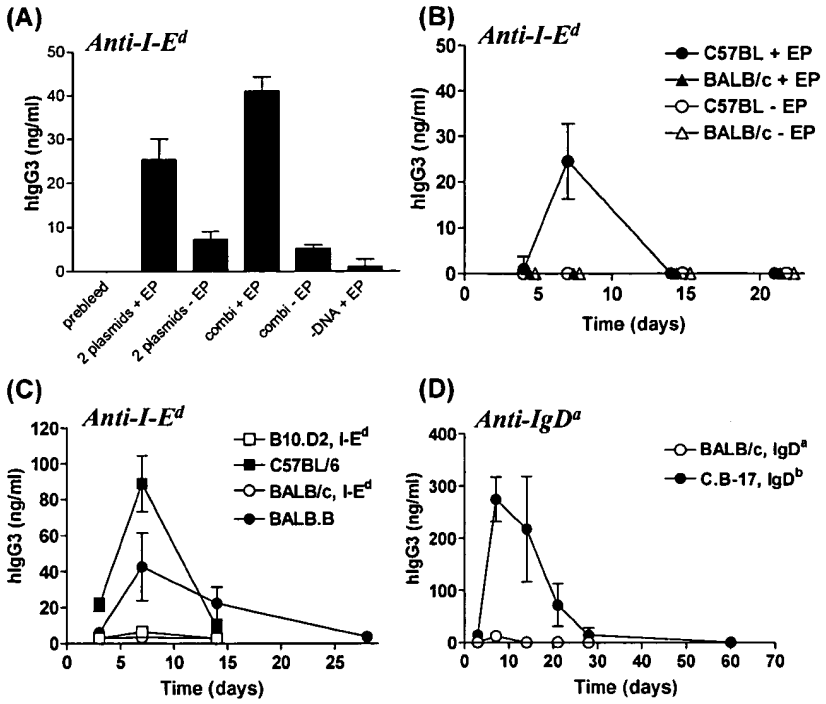

FIG. 1. Production of mAbs by intramuscular injection of Ig gene-containing plasmids, followed by *in vivo* electroporation. (A) A chimeric IgG3κ mAb with mouse V regions and human constant regions may be produced by either two separate plasmids (pLNOH2γ3V_HS and pLNOκV_LS) or a "combi" plasmid containing both H and L chain genes. The chimeric mAb has specificity for the mouse MHC class II allotype I-E^d (Table 1). Combi plasmids or equal amounts of H+L plasmids were injected im into the two quadriceps muscles (50 μg/muscle) of anesthetized C57Bl/6 mice followed (+EP) or not followed (−EP) by *in vivo* electroporation consisting of low-voltage, high-frequency electric pulses applied to the skin above the injection site. Human IgG3 in serum was measured by ELISA on day 6. (B) Combi plasmid of (A) was injected into I-E^d-negative C57Bl/6 and I-E^d-positive BALB/c mice, followed (+EP) or not followed (−EP) by electroporation. Serum levels of human IgG3 are shown. (C) Injection/electroporation of combi plasmid into paired strains of mice that have the same genetic background but which differ in the absence or presence of I-E class II molecules [BALB.B (lacks I-E) vs BALB/c (I-E^d) and C57Bl/6 (lacks I-E) vs B10.D2 (I-E^d)]. (D) Co-injection of two separate plasmids (pLNOH2γ3V_HT and pLNOκV_LT) encoding a chimeric IgD^a-specific IgG3κ mAb with mouse V regions and human constant regions, followed by electroporation. BALB/c mice express the IgD^a while the close-to-identical congenic C.B-17 mice do not express IgD^a but rather IgD^b. Each group in (A–D) consisted of three to seven mice and the bars represent the standard errors of mean.

C.B-17 mice, which lack IgD^a but express IgD^b. By contrast, in BALB/c mice, which express IgD^a but are close to identical to C.B-17, we detected no serum mAb. Serum levels of anti-IgD^a mAb reached about 300 ng/ml, which was considerably higher than that observed for the anti-I-E^d mAb.

Importantly, neither IgD nor MHC class II molecules, like I-E^d, are expressed on muscle cells, but rather by B cells, dendritic cells, and macrophages found predominantly in lymphoid organs. As the binding of mAb to antigen depends on the correct association of H and L chains, the results of Fig. 1 suggest that muscle cells secrete mAbs with correct specificity and that the mAbs reach distant tissues where they are absorbed. To investigate directly the structure of the mAb, we pooled sera from C.B-17 mice of Fig. 1D, subjected it to gel electrophoresis under reducing and nonreducing conditions, and probed the blots with antibodies against human IgG3 and human Cκ. The results show that serum mAb encoded by the plasmid consisted of disulfide-bound $(H+L)_2$ and was of a size and structure similar to those of normal IgG (Fig. 2).

Xenogeneic Parts of Chimeric Human–Mouse mAb Induce Antibody Responses

The abrupt decline in I-E^d-specific serum mAb seen in C57Bl/6 mice between days 7 and 14 (Figs. 1B and 1C) could be caused by an immune response against the xenogeneic parts of the mAb, namely human Cγ3 and human Cκ (Table 1). Indeed, the decline of anti-I-E^d mAb was matched by the presence of mouse anti-human IgG3 antibodies of both IgG1 and IgG2a subclasses, with high serum titers being detected day 28 after injection (Fig. 3). We also observed an antixenogeneic antibody response in mice that had low serum levels of the muscle-produced chimeric mAb due to absorption (Figs. 1 and 3). For unknown reasons, C.B-17 mice produced fewer anti-human IgG3 antibodies in response to muscle-produced anti-IgD^a chimeric mAb, and the IgG2a subclass was conspicuously lacking.

Sustained Expression of Syngeneic mAb

We next tested if mAb expression could be sustained if the xenogeneic parts of the mAb were reduced or abolished. First, we co-injected two separate H- and L-chain gene plasmids that together encode an IgG2b mouse antibody with human C_κ; this mAb has specificity for the mouse IgD^a allotype (Table 1). Confirming the results of Fig. 1A, C.B-17 mice injected with plasmids did not produce significant amounts of the mAb unless the injection site was also subjected to electroporation (Fig. 4A). Serum mAb reached levels as high as 750 ng/ml after 3–5 weeks and then declined slowly. Even after 7 months, mice had ~300 ng/ml in their sera. It should

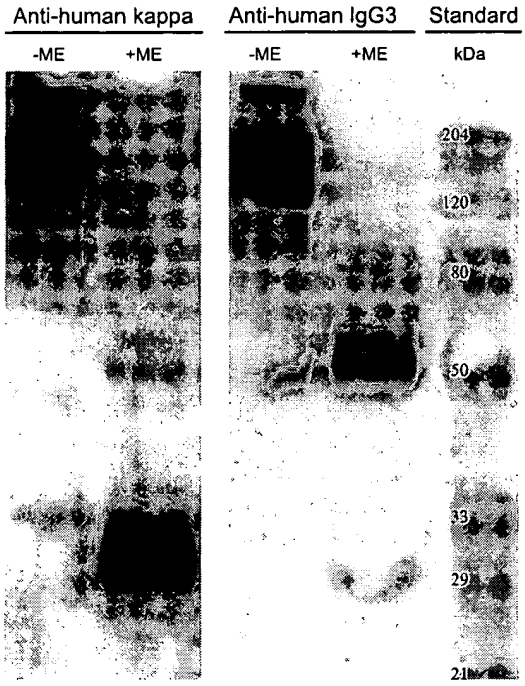

FIG. 2. Immunoblot of serum mAb produced by muscle cells. Protein G–Sepharose beads were incubated with a pool of sera from C.B-17 mice of Fig. 1D and bound IgG was eluted in SDS sample buffer. The samples were divided in two and one sample was reduced with 2-mercaptoethanol (ME) before electrophoresis. The gel was blotted onto a nitrocellulose filter that was probed with mAbs against human IgG3 and Cκ in a chemiluminescence assay.

be noted that in this experiment, we detected plasmid-encoded mAbs by their ability to bind recombinant mouse IgD^a in an ELISA; thus, only mAbs with correct specificity were detected. This experiment indicated that a reduction of xenogeneic parts greatly prolonged the presence of mAbs in serum and that the mouse IgG2b^a allotype and human Cκ are not very immunogenic in C.B-17 (IgH^b) mice.

We next examined the persistence of a fully mouse mAb by injection of two plasmids that together encode an allotype-matched mouse IgG2bλ1 mAb with specificity for NIP (Table 1). Injected and electroporated BALB/c mice had significant amounts of anti-NIP mAb in their sera measured by their ability to bind NIP-BSA in an ELISA, with maximal amounts of 60–100 ng/ml being detected between 2 and 5 weeks (Fig. 4B). In a second experiment (Fig. 4B, inset) slightly higher serum concentrations (175 ng/ml) were obtained, and we detected as much as 100 ng/ml even as late as 30 weeks after DNA injection. Electroporation was required for detection of mAb in serum (Fig. 4B). Injection of 10 and 100 μg plasmid gave similar results but 10 μg resulted in more variability in responses among mice (Fig. 4B).

Serum Anti-NIP mAb Produced by Muscle Exerts a Biological Effector Function, Complement Activation

When antibodies are aggregated on an antigenic surface, complement activation requires the presence of a disulfide bond between heavy chains and the presence of carbohydrates and paired CH2 domains. We therefore tested if the Fc region of IgG2b anti-NIP mAb produced by muscle was intact as measured by its ability to induce complement activation. We purified IgG from sera on protein A–Sepharose and added them to NIP-sensitized, ^{51}Cr-labeled sheep red blood cells in the presence of complement. IgG from mice injected with plasmids and electroporated induced complement-mediated lysis of the NIP target, while IgG from normal mice did not (Fig. 5A). Moreover, anti-NIP IgG2b in the serum IgG fraction was at least as potent as the corresponding mAb purified from supernatant of transfected cells (Fig. 5B). As negative control, an IgG1 anti-NIP mAb, which cannot activate complement [21], induced no lysis.

Anti-IgD Monoclonal Antibodies Produced by Muscle Induce a Dramatic Reduction in the Fraction of IgD-Positive B Cells in Blood and Spleen

We next tested whether the antibody produced could modulate the expression of its target *in vivo*. We injected BALB/c mice with plasmid DNA encoding mouse anti-IgD or anti-NIP, followed by electroporation. Fourteen days after the treatment, we stained blood leukocytes

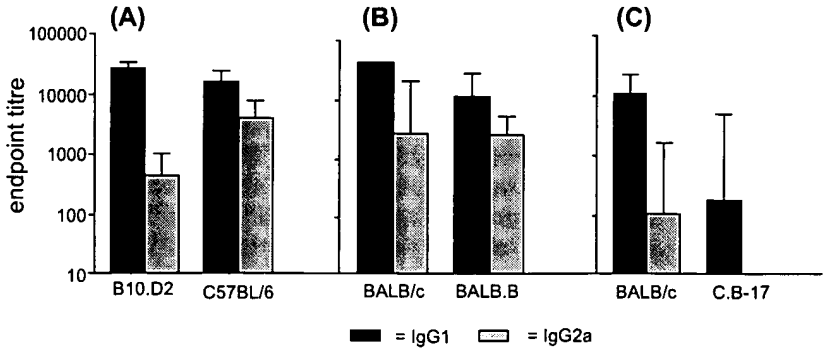

FIG. 3. Induction of mouse antibodies against xenogeneic parts of chimeric mAb encoded by injected plasmid DNA. Day 28 sera from the experiments of Figs. 1C and 1D were analyzed for mouse IgG1 (black bars) and IgG2a (gray bars) antibodies against human IgG3 in a sandwich ELISA. (A) and (B) correspond to experiments of Fig. 1C, while (C) corresponds to Fig. 1D. Results are presented as antibody endpoint titer and error bars represent standard errors of mean.

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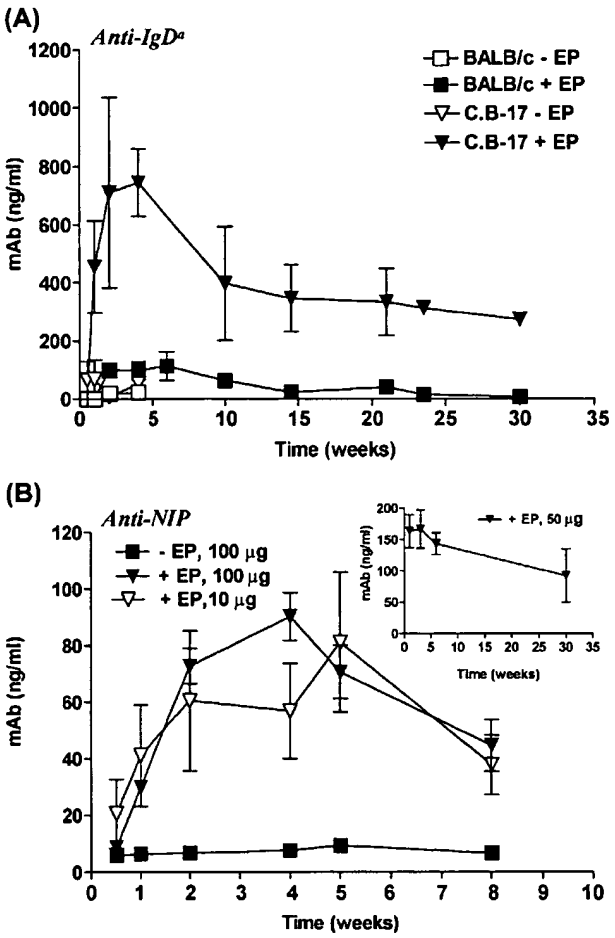

FIG. 4. Sustained production of near fully or fully mouse mAb. (A) Separate H- and L-chain gene plasmids that together encode an anti-IgD^a mAb that is fully mouse IgG2b except for human Cκ (Table 1) were co-injected in equal amounts into each quadriceps of BALB/c or C.B-17 mice (total 100 µg plasmid/mouse), followed (+EP) or not followed (−EP) by electroporation. Serum levels of IgG2b with IgD^a specificity are shown. (B) Separate H- and L-chain gene plasmids that together encode a fully mouse IgG2bλ1 anti-NIP mAb were co-injected im into BALB/c mice (100 µg or 10 µg plasmid/mouse), followed (+EP) or not followed (−EP) by electroporation. Serum levels of mouse IgG2b with NIP specificity are shown. This experiment was repeated using a total of 50 µg DNA of each plasmid and anti-NIP levels in serum were followed for 7 months (inset). Each group consisted of three to seven mice and the bars represent the standard errors of mean.

and splenocytes for B, T, and monocyte/macrophage cell markers and analyzed them by flow cytometry. Fig. 6A shows TCRβ chain vs IgD dot plots of blood lymphocytes from mice that had received either anti-NIP or anti-IgD plasmids. As can be seen, anti-IgD plasmids induced a drastic reduction of IgD$^+$ B cells, while the frequency of T cells was unchanged. The finding was statistically confirmed in a larger series of mice, and we saw similar effects in the spleen, in terms of both frequency of cells and absolute cell number (Fig. 6B and data not shown). When we treated mice of a congenic mouse strain not carrying IgD^a (C.B-17) in the same way, no reduction in IgD$^+$ cells was observed, demonstrating the allotype specificity in the reduction of IgD$^+$ B cells (data not shown). In other experiments, we tested the frequency of IgD$^+$ cells in blood on days 7, 14, 21, and 42 after treatment, and at all time points there was a significant reduction, indicating a long-lasting effect (results not shown). It should be noted that the anti-IgD mAb used in the present study does not seem to deplete B cells, as the numbers of cells labeled with the B cell marker B220 (CD45R) were unaffected (Fig. 6B). Moreover, an excess of anti-IgD^a mAb (Ig (5a)7.2) corresponding to the DNA-encoded mAb (Table 1) blocked the binding of FITC–anti-IgD to B cells during the staining procedure (results not shown). Thus, the effect of the treatment is most likely due to blocking or down regulation of IgD on B cells after binding of the mAb to its target *in vivo*.

Mouse mAb Anti-NIP Is Expressed and Secreted by Sheep Muscle

For any human clinical application, it is crucial to demonstrate that DNA injection and electroporation induce serum mAb expression in larger animals. We injected sheep weighing 15–17 kg (8–10 weeks of age) intramuscularly with 100 µg DNA encoding mouse IgG2b anti-NIP followed by electroporation. Note that due to the larger size of sheep, the equipment used for electroporation was different from that used in the mouse (see Materials and Methods). The results in Fig. 7A show that six of seven sheep had significant levels of mouse mAb with NIP specificity in serum. Maximal amounts were observed 1–2 weeks after the procedure; however, the levels of mAbs peaked at different time points in different animals. The abrupt decline in serum levels of mAb by day 28 is most likely explained by antibody responses mounted against the foreign mouse IgG2b protein (Fig. 7B). We saw the antibody responses first on day 28 and they had vanished by day 70. The induction of anti-NIP mouse IgG2b, followed by anti-mouse IgG antibodies, was specific because sheep injected with DNA encoding secreted human alkaline phosphatase (SEAP) followed by electroporation showed no such effects (Figs. 7A and 7B). Interestingly, the single sheep that failed to produce anti-NIP mAb (Fig. 7A) had high titers of sheep anti-mouse IgG Ab (not shown), indicating that although not detectable in serum, the mouse mAb was produced in sufficient amounts to induce an immune response.

DISCUSSION

We show that injection of Ig genes as naked plasmid DNA into mouse skeletal muscle, combined with electroporation of the injection site, yields correctly assembled serum mAb with intact specificity and biological effector

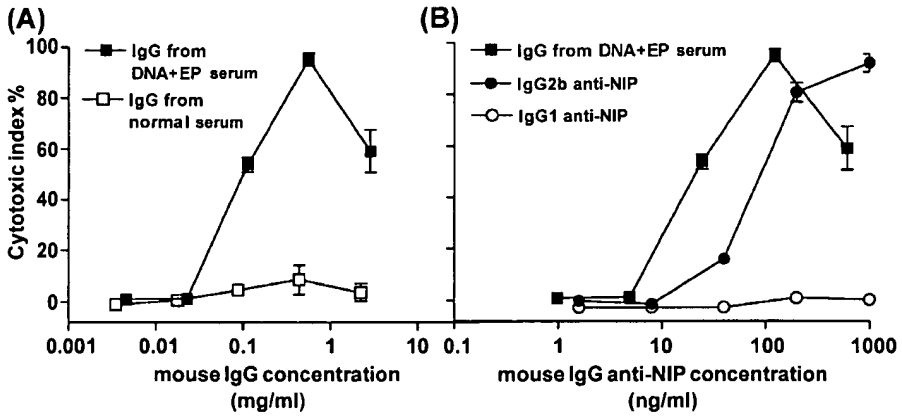

FIG. 5. Recombinant mAbs from plasmid-injected and electroporated mice induce complement activation. Sera from normal mice and mice treated with DNA encoding mouse IgG2 anti-NIP and electroporation (DNA+EP) were separately pooled, and IgG was purified on protein A–Sepharose and tested for its ability to lyse NIP-sensitized ^{51}Cr-labeled SRBC in the presence of human complement. (A) Ability of IgG of injected/electroporated mice to induce complement-mediated lysis of NIP targets compared to IgG from normal mice. (B) Anti-NIP IgG2b mAb in serum IgG fraction of DNA+EP mice was compared to anti-NIP IgG2b purified from supernatant of transfected cells in their ability to activate complement. Purified IgG1 anti-NIP, which cannot activate complement, was used as a negative control. The results are presented as the mean % cytotoxic indices of three independent experiments and the bars represent standard errors of mean.

function. The electroporation applied as low-voltage, high-frequency electrical pulses above the injection site greatly enhanced serum antibody levels. The persistence of mAb in serum for several months is most likely explained by Ig gene expression in nondividing muscle cells, which is consistent with previous findings that the vast majority of transfected cells in striated musculature are muscle cells [18,19].

Injection of H- and L- chain genes joined on the same plasmid, as well as injection of separate plasmids for the two genes, resulted in assembled $(H+L)_2$ molecules in comparable efficiencies. Thus, in multinucleated striated muscle cells, as in mononucleated transfected cells, polypeptides encoded by independent plasmids most likely pair in the endoplasmic reticulum and form disulfide bonds prior to secretion. Importantly, the V regions of

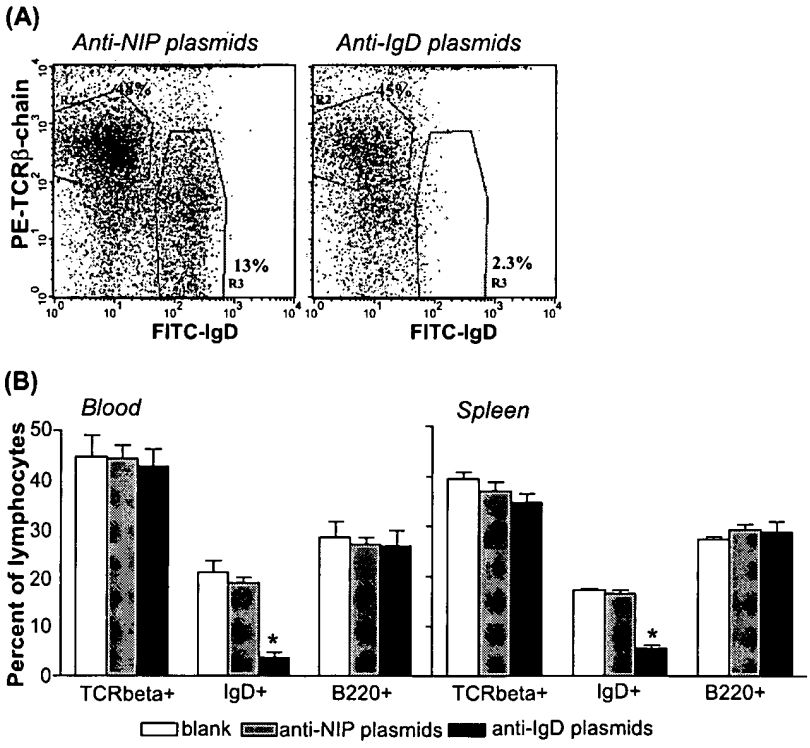

FIG. 6. Treatment of BALB/c mice with anti-IgD plasmids and electroporation reduces the frequency of IgD$^+$ B cells in blood and spleen. H and L plasmids that together encode a mouse IgG2b anti-IgD^a or anti-NIP mAb were injected into muscles of BALB/c mice (40 μg/mouse), followed by electroporation. Fourteen days later, blood and spleen cells were stained for IgD, B220/CD45R, TCRβ chain, and CD11b. (A) IgD vs TCRβ chain dot plot of blood samples from BALB/c mice receiving either anti-NIP or anti-IgD plasmids. (B) Percentages of TCR$^+$, IgD$^+$, and B220$^+$ cells among gated CD11b$^-$ lymphocytes in blood and spleen 14 days after injection of anti-NIP or anti-IgD plasmids into BALB/c mice (n = 9). Error bars represent the standard errors of the mean; a statistically significant result (P< 0.05) is indicated by an asterisk.

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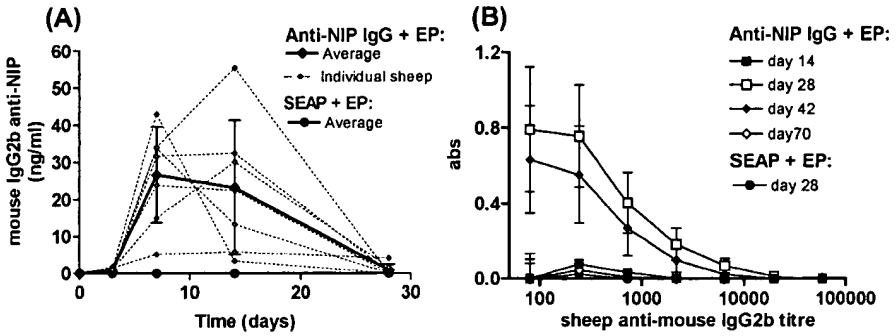

FIG. 7. Sheep muscle produces mouse anti-NIP mAb. Sheep (15–17 kg, n = 7) were injected at day 0 with an equal mixture of pLNOH2γ2bV$_H$NIP and λ1 plasmids (100 μg DNA) and electroporated at the site of injection ("anti-NIP IgG + EP"). As a control, sheep (n = 7) received DNA encoding human alkaline phosphatase followed by electroporation ("SEAP + EP"). (A) For sheep treated with anti-NIP IgG + EP, concentrations of mouse anti-NIP mAb in serum at the indicated time points are shown for each individual as well as on average. For sheep treated with SEAP + EP, only the average is given. (B) Sheep anti-mouse IgG2b antibody titers are shown for the indicated time points. The bars represent standard errors of mean.

the mAbs were correctly folded because in all instances they had the expected specificity for NIP, IgD^a, or I-E^d. Also the Fc regions seemed to have a functional glycosylation pattern since the muscle-produced mAbs had the ability to activate complement.

Electroporation-mediated DNA vaccination has previously been used to elicit immune responses against foreign proteins and is an attractive strategy for active vaccination [22,23]. Consistent with these previous results, we found that if the plasmid-encoded mAb had a xenogeneic constant heavy chain, antixenogeneic Abs were readily induced in both mice and sheep; as a consequence, the mAb persisted in serum for only a few weeks. By contrast, a fully mouse mAb was expressed in serum at high levels for as long as 7 months. Small amounts of antigenic material, such as the variable regions of a different mouse strain, the mouse IgG2b^a allotype, or the human Cκ, were apparently accepted without seriously compromising long-term serum mAb expression. Since H- and L-chain genes encoding a fully sheep mAb are not available, we were unable to test longevity of muscle-produced mAb in this animal.

It has previously been shown that inclusion of xenogeneic Ig constant regions [24] or tetanus toxoid fragment C [25] induces so-called anti-idiotypic (Id) antibodies that bind to the V regions of muscle-produced mAb. For the purpose of therapeutic mAb expression, which is the focus of the present work, induction of anti-Id Ab is unwanted since such Ab would block antigen binding sites on muscle-produced Ig and reduce its serum levels. Moreover, immune complexes of anti-Id and muscle-produced mAb could induce pathology and disease. It is therefore encouraging that expression of fully mouse Ig genes in the absence of foreign sequences led to persistent high serum levels of NIP-specific mouse IgG2b and that no anti-Id antibodies were detected (results not shown). Therefore, for clinical purposes, fully human genes should probably be used; such genes could be obtained from human single-chain F$_V$ and Fab fragment phage display libraries or from mice engineered to express only human Ig genes.

In the mouse, levels of serum IgG2b mouse mAbs in the 50–800 ng/ml range were stably present in serum for more than 7 months. Because mouse IgG2b has a half-life of 4–6 days, this finding indicates that transfected muscle cells continue to secrete Ig molecules for long periods of time after a single injection of DNA. It is promising that muscle was able to produce sufficient anti-IgD mAbs to block or down regulate IgD on B cells for at least 6 weeks. It should be possible to increase the level of mAb further by increasing the numbers of muscle sites injected and by generating vectors optimized for expression of Ig in muscle.

Passive antibody therapy of humans is a rapidly expanding field. Importantly, we could scale up the method to sheep that weighed about 25% of adult humans. Serum levels of 30–50 ng/ml were obtained, which amount to about 0.2 mg mAb present in a sheep at any time point. This level of mAb was reached with only 100 μg DNA and using a mouse construct that led to immune reactions and supposedly lower levels of mAb than could be obtained using genes encoding a fully sheep mAb. Taking these considerations into account, it might well be that DNA injection and electroporation of humans could induce serum concentrations that could substitute for injection of protein mAb in treatment of diseases like B lymphomas (anti-CD20 mAb [3]), breast cancer (anti-Her 2 mAb [26]), and rheumatoid arthritis (anti-TNFα mAb [5]). However, an application to humans might necessitate the use of inducible promoters so that expression can be turned off when the mAb is no longer needed.

Plasmid DNA injection and *in vivo* electroporation are simple, are cheap, carry minimal risk of inadvertent infection, and appear to be nontoxic. No detrimental effects have yet been observed in larger animals, like goat and cattle that have undergone this procedure under local anesthesia [27] and sheep that were not anesthetized (present study). Scaling up from mice to human should

therefore be feasible. If so, sustained expression of muscle-produced mAb might become a substitute for repetitive injections of protein mAb and could be of value in antibody-based treatment of chronic illnesses like cancer and autoimmune diseases.

Aside from mAb therapy, DNA injection and electroporation of mouse muscle could be a valuable method for rapid testing of new Ig constructs and their efficacies *in vivo*. Moreover, the method might be used for production of Ig fragments like single-chain Fv, which for certain therapeutic purposes could represent an advantage compared to complete Ig molecules.

MATERIALS AND METHODS

Animals and Electroporation of Muscle

Mice. Five- to ten-week-old BALB/c, C57Bl/6 (B&K Universal AB, Sweden), B10.D2, BALB.B (Harlan, England), and C.B-17 (M&B, Denmark) mice were used. The mice were anesthetized by intraperitoneal injection with 9 μg pentobarbital/mouse and the legs were shaved. Conductive gel was applied at the skin and 10–50 μg vector DNA, diluted in 50 μl 0.9% NaCl, was injected into each of the quadriceps muscles. Immediately following injection, electroporation was performed using a caliper electrode as previously described [23].

Sheep. Seven outbred sheep weighing 15–17 kg were injected intramuscularly with vector DNA in the gluteus muscle and electroporated using an invasive two-needle electrode (Inovio AS, Oslo, Norway) with a distance between the needles of 8 mm. Six pulses of 20 ms length with an amplitude of 250 mA and about 50 V/cm were applied at the site of DNA injection (100 μg of each plasmid/100 μl 0.9% NaCl). The electrical field was generated by a pulse generator constructed for the purpose (Inovio AS).

Plasmids

The plasmids employed are based on two expression vectors constructed for easy exchange of the variable and constant parts of Ig H-chain genes (pLNOH2) and Ig L-chain genes (pLNOκ) [28]. Plasmids and the recombinant mAb they encode are described in Table 1. V regions were derived from mouse B cell hybridomas, while constant parts were either of mouse (Cγ2b, Cλ) or human (Cγ3, Cκ) origin (Table 1). The pLNOH2γ2bV_HT vector was constructed by replacing the human γ3 constant region gene of pLNOH2γ3V_HT with mouse γ2b [29,30] (unpublished). Separate plasmids for H-chain and L-chain genes were used except for the I-E^d specificity, for which a single combi vector encoding both H- and L-chain genes [31] was used in some experiments. Ig genes were under the control of a CMV promoter. The plasmids were purified according to the procedure of the Concert High Purity Plasmid Maxiprep System (Life Technologies, UK).

ELISA

Levels of mAb in serum were determined by ELISA. Wells were coated with a capture protein after which diluted sera were added. Biotinylated detection antibodies, streptavidin–alkaline phosphatase (Amersham Pharmacia Biotech UK Lt., UK), and *para*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, USA) were added sequentially. The following sandwich ELISAs were used: For measurement of chimeric human IgG3 anti-I-E^d and anti-IgD, anti-human IgG3 (I-9763, Sigma) was used as coat antibody and biotinylated anti-human IgG3 (B-3523, Sigma) was used as detection antibody. For measurement of mouse IgG2b anti-NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid), $NIP_{2.6}$BSA (i.e., 2.6 NIP molecules per BSA molecule) was used as a coat antigen and biotinylated anti-mouse IgG2b (Pharmingen, BD Biosciences) was used as detection antibody. For measurement of mouse IgG2b anti-IgD, an IgD coat was obtained by first coating the wells with $NIP_{2.6}$BSA followed by mouse IgD anti-NIP. Biotinylated anti-mouse IgG2b (Pharmingen) was used as detection antibody. Standard curves were constructed with anti-NIP [29] or anti-IgD mAbs (affinity purified from cell transfectants) or with anti-human IgG3 (Sigma). For determination of mouse anti-human IgG3 titer, human IgG3 (Sigma) was used as coat and biotinylated anti-mouse IgG1 or anti-mouse IgG2a (both Pharmingen) were used as detection antibodies. For determination of sheep anti-mouse IgG2b titer, mouse IgG2b (affinity purified from cell transfectants) was used as coat and biotinylated rabbit anti-sheep IgG was used as detection antibody.

Gel Electrophoresis and Blotting

Serum from mice that had been injected with DNA plasmids and electroporated were incubated with protein G–Sepharose beads (Sigma). The bound antibodies were eluted in SDS sample buffer. The eluted samples were divided in two and in one sample 2-mercaptoethanol was added to reduce disulfide bonds between the H and the L chains. After SDS–PAGE (10%) and transfer to nitrocellulose filters, the blots were incubated with either biotinylated anti-human IgG3 (B-3523, Sigma) or biotinylated anti-human κ (B-1393, Sigma). The results were visualized by chemiluminescence assay (ECL, Amersham Pharmacia Biotech).

Complement-Mediated Cell Lysis (CML)

The CML assay was performed as described [32]. Briefly, ^{51}Cr-labeled sheep red blood cells (SRBC) were incubated with NIP-conjugated rabbit anti-SRBC Fab′ fragments and washed. For 1×10^8 SRBC a total of 400 ng NIP-Fab was used with an average of 15 hapten molecules per Fab (NIP_{15}Fab). Serial dilutions of IgG purified from mouse serum on protein A–Sepharose by standard procedures (Amersham Bioscience) were then added to NIP-sensitized ^{51}Cr SRBC. Human serum was used as a source of complement. As negative control, mouse IgG1 anti-NIP was used (clone N1-G9, kindly provided by M. Neuberger [21]). IgG2b anti-NIP from cell transfectants was used as a positive control. The cytotoxic index (CI) was calculated according to the formula %CI = [(cpm test − cpm spontaneous)/(cpm max − cpm spontaneous)] × 100.

Flow Cytometry

Blood from the tail vein was collected in heparinized tubes. Single-cell spleen suspensions were made by squeezing spleens through a stainless steel grid. Red blood cells were lysed with lysis buffer (0.75% (w/v) NH_4Cl, pH 7.2). Fc receptors were blocked by incubation with 30% heat-inactivated normal rat serum and 100 μg/ml 2.4G2 (monoclonal antibody anti-Fcγ II/III receptor, ATCC) for 15 min on ice prior to staining with fluorochrome-labeled mAbs (diluted in PBS with 0.5% BSA) for 15 min on ice. Fluorochrome-labeled mAbs were FITC-11-26c.2a (anti-IgD), PerCP-RA3-6B2 (anti-B220/CD45R), APC-M1/70 (anti-CD11b) (PharMingen, USA); and PE-H57-597 (anti-TCRβ chain) (Southern Biotech, Birmingham, AL, USA). Quadruple-stained cells were analyzed by a FACSCalibur and Cellquest software (Becton Dickinson). Cells within a typical forward/side scatter lymphocyte gate were selected for further analysis and the percentage of cells positive for the different markers was calculated.

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